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<b>(21) International Application Number:</b> PCT/GB97/00577 <b>(22) International Filing Date:</b> 3 March 1997 (03.03.97)  <b>(30) Priority Data:</b> 9604480.5 1 March 1996 (01.03.96) GB 9605598.3 16 March 1996 (16.03.96) GB  <b>(71) Applicant (for all designated States except US):</b> THE UNIVERSITY COURT OF THE UNIVERSITY OF DUNDEE [GB/GB]; Tower Building, Dundee DD1 4HN (GB).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> BURCHELL, Brian [GB/GB]; 8 Dougall Street, Tayport, Fife DD6 9 JB (GB).  <b>(74) Agent:</b> MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> DRUG TRIAL ASSAY SYSTEM  <b>(57) Abstract</b>  The invention provides a method for improving the efficacy of drug trials, the method comprising the step of screening samples from potential participants for the genetic basis of Gilbert's Syndrome and eliminating or including potential participants in a drug trial in the knowledge of them possessing or not possessing the genetic basis of Gilbert's Syndrome.		

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1 "Drug Trial Assay System"

2

3 The present invention relates to drug trials, usually  
4 carried out for or on behalf of pharmaceutical  
5 companies. More particularly the invention relates to  
6 a method for improving the efficacy of drug trials.

7

8 In the different stages of drug trials, regulatory  
9 authorities in different European countries and the FDA  
10 in the USA require extensive data to be provided in  
11 order to approve use of the drugs.

12

13 It is important that as much information as possible is  
14 available in relation to all participants who take part  
15 in drug trials, from volunteers who take part in phase  
16 1 trials to patients involved in stage 3 clinical  
17 trials.

18

19 In particular, if certain individuals or groups of  
20 individuals have severe or abnormal reactions to drug  
21 administration, further studies involving that drug  
22 will be in jeopardy unless the reason for the reaction  
23 is realised.

24

25 The knowledge of pharmacogenetics can play an important

1 role in understanding the impact of drug metabolism on  
2 pharmacokinetics, role of receptor variants in drug  
3 response and in the selection of patient populations  
4 for clinical studies.

5  
6 Considerable effort has been expended in attempting to  
7 identify the pharmacogenetic basis of idiosyncratic  
8 adverse drug reactions, particularly hypersensitivity  
9 reactions. While there is clear evidence for  
10 pharmacogenetic influence on susceptibility to  
11 hypersensitivity reactions, necessary and sufficient  
12 pharmacogenetic defects have not been identified.

13  
14 The clinical implications of genetic polymorphism in  
15 drug metabolism have been studied extensively (See  
16 Tucker GT (1994) Journal Pharmacology 46 pages 417-  
17 424).

18  
19 Gilbert's Syndrome (GS) is a benign unconjugated  
20 hyperbilirubinaemia occurring in the absence of  
21 structural liver disease and overt haemolysis and  
22 characterized by episodes of mild intermittent  
23 jaundice. It is part of a spectrum of familial  
24 unconjugated hyperbilirubinaemias including the more  
25 severe Crigler-Najjar (CN) syndromes (types 1 and 2).  
26 GS is the most common inherited disorder of hepatic  
27 bilirubin metabolism occurring in 2-12% of the  
28 population and is often detected in adulthood through  
29 routine screening blood tests or the fasting associated  
30 with surgery/intercurrent illness which unmasks the  
31 hyperbilirubinaemia<sup>1,3</sup>. The most consistent feature in  
32 GS is a deficiency in bilirubin glucuronidation but  
33 altered metabolism of drugs has also been reported<sup>3,5</sup>.  
34 Altered rates of bilirubin production, hepatic haem  
35 production and altered hepatic uptake of bilirubin have  
36 been reported in some GS patients<sup>2</sup>.

1 Due to the benign nature of the syndrome and its  
2 prevalence in the population it may be more appropriate  
3 to consider GS as a normal genetic variant<sup>2</sup> exhibiting a  
4 reduced bilirubin glucuronidation capacity (which in  
5 certain situations such as fasting, illness or  
6 administration of drugs) could precipitate jaundice.

7  
8 In drug trials where high levels of serum total  
9 bilirubin is detected for certain individuals, it is  
10 not clear whether this is because the individuals have  
11 Gilbert's Syndrome or if it because of an effect of the  
12 drug. Whereas presently, results are explained merely  
13 by saying that the individuals have Gilbert's Syndrome,  
14 it is suspected that in the future, it will be  
15 necessary to prove this fact.

16  
17 Where a jaundiced phenotype is apparent after  
18 volunteers have been accepted for a trial and have been  
19 subjected to five days of a strict diet, no alcohol and  
20 no smoking, the jaundiced appearance giving an  
21 indication that the individuals have Gilbert's  
22 Syndrome, may cause them to be ruled out of the trials.  
23 Therefore, where approximately 250 individuals would be  
24 required for phase 1 trials and about 6000 patients for  
25 phase 3 trials, unnecessary time and effort would have  
26 been spent during the first 5 days of these trials and  
27 individuals having Gilbert's Syndrome may be ill  
28 effected.

29  
30 The present invention aims to provide a method of  
31 improving the efficacy of drug trials in view of the  
32 problems mentioned above.

33  
34 According to the present invention there is provided a  
35 method for improving the efficacy of drug trials, the  
36 method comprising the step of screening samples from

1 individuals for the genetic basis of Gilbert's  
2 Syndrome.

3

4 In a preferred embodiment of the invention the method  
5 comprises the steps taking a sample from each potential  
6 participant in a drug trial, screening the samples for  
7 the genetic basis of Gilbert's Syndrome, identifying  
8 participants having the genetic basis of Gilbert's  
9 Syndrome.

10

11 The sample may comprise blood, a buccal smear or any  
12 other sample containing DNA from the individual to be  
13 tested.

14

15 In one embodiment the method comprises the further step  
16 of eliminating participants having the genetic basis of  
17 Gilbert's Syndrome from the drug trial.

18

19 In an alternative embodiment, the method can comprise  
20 the further step of selecting participants having the  
21 genetic basis of Gilbert's syndrome and eliminating  
22 others from the drug trial.

23

24 In a further alternative the results of the drug trials  
25 can be interpreted in the knowledge that certain  
26 participants have Gilbert's Syndrome.

27

28 Preferably the method comprises the steps of isolating  
29 DNA from each sample, amplifying the DNA in a region  
30 indicating the genetic basis of Gilbert's Syndrome,  
31 isolating amplified DNA fragments by gel  
32 electrophoresis and identifying individuals having the  
33 genetic basis of Gilbert's disease.

34

35 Preferably the DNA is amplified using the polymerase  
36 chain reaction (PCR) using a radioactively labelled

1 pair of nucleotide primers.

2

3 The primers are designed to prime the amplification  
4 reaction at either side of an area of the genome known

5

6 to be associated with Gilbert's Syndrome.

7

8 Preferably the DNA region indicating the genetic basis  
9 of Gilbert's Syndrome is the gene encoding UDP-  
10 glucuronosyltransferase (UGT).

11

12 By gene is meant, the non coding and coding regions and  
13 the upstream and downstream noncoding regions.

14

15 In a preferred embodiment the DNA to be amplified is in  
16 an upstream promoter region of the UGT1\*1 exon1.

17

18 Most preferably the DNA to be amplified includes the  
19 region between -35 and -55 nucleotides at the 5' end of  
20 UGT1\*1 exon.

21

22 According to the invention there are provided suitable  
23 primers for use in a PCR reaction including primer  
24 pairs;

25

26 A/B (A, 5'-AAGTGAAGTCCCTGCTACCTT-3',

27 B, 5'-CCACTGGGATCAACAGTATCT-3') or

28 C/D (C, 5'-GTCACGTGACACAGTCAAAC-3';

29 D 5'-TTTGCTCCTGCCAGAGGTT-3')

30

31 The invention further comprises a kit for screening  
32 individuals for participation in drug trials, the kit  
33 comprising primers for amplifying DNA in a region of  
34 the genome indicating the genetic basis of Gilbert's  
35 Syndrome.

36

1 Using primer sequences as described herein, DNA can be  
2 amplified and analysed using among others any of the  
3 following protocols;

4

5 Protocol 1 Radioactive method

6

7 1. Extract DNA from Buccal Cells or 3ml Blood.

8

9

10 2. Choose primers from either side of the "TATA" box  
11 region of UGT1\*1 exon1 regulatory sequence.

12 Freshly end label one primer with [ $\gamma$   $^{32}\alpha$ ]-ATP (40  
13 min).

14

15 3. Amplifying a small region up to 100 bp in length  
16 by PCR (2h).

17

18 4. Apply to 6% PAG denaturing gel (preparation,  
19 loading, run time, 4h).

20

21 5. Expose (-70°C) wet gel to autoradiographic film  
22 (15 min).

23

24 This method takes about 7h to complete. Polymorphisms  
25 only observed in TATA box non coding region todate.

26

27 Protocol 2

28 Alternative Radioactive Method: Solid Phase

29 Minisequencing

30

31 1. Extract DNA (as above)

32

33 2. Prepare primers biotinylating one

34

35 3. Amplify DNA by PCR using primers

36



1 4. Captive biotinylated PCR products on streptavidin  
2 coated support and deactive.

3

4 5. Carry out primer extension reaction sequencing.

5

6 Protocol 3

7 Non-Radioactive Methods:

8

9 (a) Analysis by Single Strand Conformational  
10 Polymorphism (SSCP)

11 1. Extract DNA (as above).

12

13 2. Choose primers either side of the TATA Box.

14

15 3. Amplify a small region up to 100 bp in length by  
16 PCR (2H).

17 4. Denature and place on ice (15 min).

18

19 5. Load onto a non-denaturing PAG gel,  
20 (preparation/load/run time, 4h).

21

22 6. Stain with Ethidium bromide or silver nitrate (30  
23 mm).

24

25 This method still takes about 7h to complete, but is  
26 potentially slightly cheaper since there is no  
27 radioactivity or autoradiography.

28

29 This method could be done on an automated DNA sequencer  
30 from stage 5, if primers are tagged with chromophores  
31 in PCR stages 2 and 3. Result would then be read  
32 automatically.

33

34 (b) Oligonucleotide Assay Hybridization

35

36 1. Extract DNA (as above).

- 1     2.    Choose primers and amplify DNA by PCR up to 100 bp
- 2            in length.
- 3
- 4     3.    Apply DNA to plastic grids.
- 5
- 6     4.    Screen bound DNA samples with specific DNA probes
- 7            for TA<sub>5</sub>, TA<sub>6</sub>, TA<sub>7</sub> tagged with different
- 8            coloured/fluorescent chromophores.
- 9
- 10    5.    Read output automatically for experimental
- 11           protocols.
- 12
- 13    References
- 14
- 15    Monaghan G et al. Lancet (1996) 347 578-581.
- 16
- 17    "Detection of polymorphisms of human DNA by gel
- 18    electrophoresis or single-strand conformational
- 19    polymorphisms"." Orita M et al. Proc Natl Acad Sci
- 20    (USA) (1989) 86 2766-2700.
- 21
- 22    "Assays of complementary oligonucleotides for analysing
- 23    Hybridization behaviour of Nucleic Acids". Southern E
- 24    M. Nuc Acids Res (1994) 22 1368-1373.

1 The basis of the invention is illustrated in the  
2 following example with reference to the accompanying  
3 figures wherein:

4  
5 Figure 1 illustrates genotypes at the TATA box sequence  
6 upstream of the UGT1\*1 exon 1 determined by direct  
7 sequencing and radioactive PCR.

8  
9 Figure 2 illustrates serum total bilirubin ( $\mu\text{mol/l}$ )  
10 plotted against UGT1\*1 exon 1 genotype.

11  
12 Figure 3 illustrates segregation of the 7/7 genotype  
13 with elevated serum total bilirubin concentration in a  
14 family with GS.

15  
16 Figure 4 illustrates the 5' sequence of the UGT1\*1 exon  
17 1 and the position of the primers with respect to the  
18 UGT gene.

19  
20 Example

21  
22 We have examined the variation in the serum total  
23 bilirubin (STB) concentration in a representative group  
24 of the Eastern Scottish population (drug-free, alcohol-  
25 free non-smokers) in relation to genotype at the UDP-  
26 glucuronosyltransferase subfamily 1 (UGT1) locus.  
27 Subjects with the 7/7 genotype in this population have  
28 a significantly higher STB than those with 6/7 or 6/6  
29 genotypes. Of 14 control subjects who underwent a 24  
30 hour fast to establish whether they had Gilbert  
31 Syndrome (GS), only 7/77 subjects had GS. In addition,  
32 one confirmed GS patient, two recurrent jaundice  
33 patients and 9 clinically diagnosed GS patients had the  
34 7/7 genotype. Segregation of the 7/7 genotype with  
35 elevated STB concentration has also been demonstrated  
36 in a family of 4 Gilbert members. This incidence of

1 the 7/7 genotype in the population is 10-13%. Here, we  
2 demonstrate a correlation between variation in the  
3 human STB concentration and genotype at a TATA sequence  
4 upstream of the UGT1\*1 exon 1 and that the 7/7 genotype  
5 is diagnostic for GS.

6  
7 The inheritance of GS has been described as autosomal  
8 dominant or autosomal dominant with incomplete  
9 penetrance based on biochemical analysis<sup>6</sup>. More recent  
10 reports have suggested that the mildly affected  
11 (Gilbert) members of families in which CN type 2 (CN-2)  
12 occurs are heterozygous for mutations in the UDI<sup>3</sup>-  
13 glucuronosyltransferase subfamily 1 (UGT1) gene which  
14 cause CN-2 in the homozygous state. The inheritance of  
15 GS in these families is autosomal dominant while CN-2  
16 is autosomal recessive<sup>7-11</sup>. However, the incidence of  
17 CN-2 in the population is very rare and the frequency  
18 of alleles causing CN-2 would not be sufficient to  
19 explain the population incidence of GS.

20  
21 An abstract by Bosma et al<sup>12</sup> suggested a correlation  
22 between homozygosity for a 2bp insertion in the TATA  
23 box upstream of UGT1\*1 exon 1 and GS (no mutations were  
24 found in the coding sequence of the UGT1\*1 gene). In  
25 this report we demonstrate that the primary genetic  
26 factor contributing to the variation in the serum total  
27 bilirubin (STB) concentration in the Eastern Scottish  
28 population is the sequence variation reported by Bosma  
29 et al<sup>12</sup>. In addition, we show that the 7/77 genotype is  
30 associated with GS and occurs in 10-13% of the  
31 population.

32

### 33 Methods

#### 34 Patients and Controls

35 Whole blood (3ml) was collected into EDTA(K3)

36 Vacutainer tubes (Becton Dickinson) from one confirmed

1 male Gilbert patient (diagnosed following a 48 hour  
2 restricted diet<sup>13</sup>), two female patients with recurrent  
3 jaundice/associated elevated STB (29-42  $\mu\text{mol/l}$ ) and 9  
4 (1 female, 8 male) clinically diagnosed GS subjects  
5 (persistent elevation of the STB amidst normal liver  
6 function tests.) The patients were aged 22-45 years.

7  
8 77 non-smoking residents selected at random from the  
9 Tayside/Fife region of Scotland (39 females aged 19-58  
10 years, mean  $32.41 \pm 10.94$ ; 38 males aged 23-57, means  
11  $35.58 \pm 9.04$ ) participated in this study. Whole blood  
12 (9ml) was collected 8-10am into EDTA(K3) Vacutainer  
13 tubes (Becton Dickinson) for DNA extraction and SST  
14 Vacutainer tubes (Becton Dickinson) for biochemical  
15 investigations. The subjects had not taken any  
16 medication or alcohol in the previous 5-7 days and had  
17 fasted overnight (12 hours). 14 controls subsequently  
18 underwent further biochemical tests (following a 3 day  
19 abstinence from alcohol) before and after a 24 hour  
20 400-calorie diet<sup>14</sup> to determine if they had GS. All  
21 patients/controls were fully informed of the study and  
22 gave consent for their blood to be used in this study.

23

#### 24 Biochemistry and DNA Extraction

25

26 The following biochemical tests were performed on  
27 control blood samples; alanine aminotransferase,  
28 albumin, alkaline phosphatase, amylase, STB,  
29 cholesterol, creatinine, creatine kinase, free  
30 thyroxine, gamma-glutamyl-transferase, glucose, HDL-  
31 cholesterol, HDL-cholesterol/total cholesterol, iron,  
32 lactate dehydrogenase, percentage of saturated  
33 transferrin (PSAT), proteins, serum angiotensin  
34 converting enzyme, thyroid stimulating hormone,  
35 transferrin, triglycerides, urate, urea. 14 controls  
36 also had pre- and post-fasting (24 hour) alanine

1 aminostransferase, albumin, alkaline phosphatase, STB  
2 and urate measured. DNA was prepared using the Nucleon  
3 II Genomic DNA Extraction Kit (Scotlab) according to  
4 manufacturer's instructions.

5

#### 6 Genotyping

7

#### 8 Polymerase Chain Reaction

9

10 Primer pairs A/B (A, 5'-AAGTGAAGTCCCTGCTACCTT-3'; B,  
11 5'-CCACTGGGATCAACAGTATCT-3') or C/D (C, 5'-  
12 GTCACGTGACACAGTCAAAC-3'; D, 5'-TTTGCTCCTGCCAGAGGTT-3')  
13 flanking the TATA box sequence upstream of the UGT1\*1  
14 exon 1 were used to amplify fragments of 253-255bp and  
15 98-100bp, respectively. Amplifications (50µl) were  
16 performed in 0.2mM of each deoxynucleoside triphosphate  
17 (dATP, dCTP, dGTP, dTTP), 50mM KCl, 10mM Tris.HCl (pH  
18 9.0 at 25°C), 0.1% Triton X-100, 1.5mM MgCl<sub>2</sub>, 0.25µM of  
19 each primer, 1 Unit of Taq Polymerase (Promega) and  
20 human DNA (0.25-0.5µg). The polymerase chain reaction  
21 (PCR) conditions using the Perkin-Elmer Cetus DNA  
22 Thermal Cycler were: 95°C 5 min followed by 30 cycles  
23 of 95° 30 sec, 58°C 40 sec, 72°C 40 sec.

24

#### 25 Direct Sequencing

26

27 Amplification was confirmed prior to direct sequencing  
28 by agarose gel electrophoresis. Sequencing was  
29 performed using [ $\alpha$ -<sup>35</sup>S]-dATP (NEN Dupont) with the USB  
30 Sequenase™ PCR Product Sequencing Kit according to  
31 manufacturer's instructions. Sequenced products were  
32 resolved on 6% denaturing polyacrylamide gels. The  
33 dried gels were exposed overnight to autoradiographic  
34 film prior to developing.

35

#### 36 Radioactive PCR

1 Amplification was performed as above using primer pair  
2 C/D except that 2.5 pmol of primer C was radioactively  
3 5' end-labelled with 2.5  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]-ATP (NEN Dupont)  
4 prior to amplification. Products were resolved on 6%  
5 denaturing polyacrylamide gels and the wet gels exposed  
6 to autoradiographic film (-70°C 15 min) and the  
7 autoradiographs developed.

8

#### 9 Statistics

10

11 A t-test was used to determine if there was a  
12 significant age difference between males and females.  
13  $\chi^2$  analysis was used to assess any difference in the  
14 distribution of the 6/6, 6/7 and 7/7 genotypes in males  
15 and females and also to determine if the 7/7 subjects  
16 from the 24 hour fasted group had STB elevated into the  
17 range diagnostic for GS<sup>14</sup>. An analysis of variance was  
18 performed to compare mean STB in males and females  
19 within each genotype group. A non-parametric test, the  
20 Mann-Whitney U-Wilcoxon Rank Sum W Test was used to  
21 determine whether there was a significant difference in  
22 mean STB between males and females (irrespective of  
23 genotype). Correlations and significance tests were  
24 performed for STB versus PSAT and STB versus iron. A  
25 probability (p) of  $\leq 0.05$  was accepted as significant.

26

#### 27 Results

28

29 In Figure 1 a photographic representation of the sense  
30 DNA sequences obtained by PCR/direct sequencing of DNA  
31 samples having the genotypes 6/6, 6/7 and 7/7 is shown.  
32 The common allele, (TA)<sub>6</sub>TAA, is denoted by "6" while the  
33 rarer allele, (TA)<sub>7</sub>TAA, is denoted by "7". Below each  
34 sequence is an overexposed photographic representation  
35 of the 98 to 100bp resolved fragments amplified using  
36 primer pair C/D which flank the TATA sequence upstream

1 of the UGT1\*1 exon 1. The additional fragments of 99  
2 and 101 bases are thought to be artifacts of the PCR  
3 process where there is non specified addition of an  
4 extra nucleotide to the 3' end of the amplified  
5 product<sup>21</sup>. Figures 1b illustrates results after testing  
6 a range of unknown individuals.

7  
8 In Figure 2 males (M) and females (F) are plotted  
9 separately. Each circle/square represents the result  
10 of a single control subject. The squares indicate the  
11 14 controls who also underwent the 24 hour restricted  
12 diet (see Methods). The filled circles/squares  
13 represent those who had a lower than normal PSAT ( $\leq$   
14 22%) while the half-tone circles represent those who  
15 had a higher than normal PSAT ( $\geq 55\%$ ). The mean STB  
16 concentrations (indicated by the horizontal lines) for  
17 males were  $13.24 \pm 3.88$  (6/6),  $13.94 \pm 6.1$  (6/7)  
18 including control h or  $12.69 \pm 3.34$  excluding control  
19 h,  $29 \pm 14.45$  (7/7) and for females were  $9 \pm 3.62$   
20 (6/6),  $12.2 \pm 3.53$  (6/7),  $21.6 \pm 7.8$  (7/7). The  
21 encircled result is from control h (discussed in the  
22 text).

23  
24 In Figure 3 males and females are represented by  
25 squares and circles, respectively. Filled and half-  
26 filled circles/squares indicate the genotypes 7/7 and  
27 6/7, respectively. The numbers in parentheses below  
28 each member of the pedigree are the STB concentrations  
29 measured after a 15 hour fast and 7 day abstinence from  
30 alcohol. All family members were non smokers who were  
31 not taking any medication when the biochemical tests  
32 were performed. Elevated STB are underlined.  
33 Individual members of each generation (I or II) are  
34 denoted by the numbers 1-4 above each circle/square.  
35 Generation III have not yet been tested.

36



1 There was no significant age difference between males  
2 and females ( $t = -1.38$ ,  $p = 0.17$ ). Genotypes were  
3 determined initially by amplification/sequencing and  
4 later by the radioactive PCR approach. Individuals  
5 homozygous for the common allele, heterozygous or  
6 homozygous for the rarer allele have the genotypes 6/6,  
7 6/7 and 7/7, respectively. 12 DNA samples (2 of 6/6, 3  
8 of 6/7 and 4 of 7/7) were analysed by both methods and  
9 genotype results were identical (see Figure 1).  
10  
11 Genotype frequencies in male controls were 6/6 (44.74%,  
12 6/7 (44.74%), 7/7 (10.52%) and in female controls were  
13 6/6 (35.9%), 6/7 (51.3%), 7/7 (12.8%). There was no  
14 significant difference between the genotype proportions  
15 in the two groups ( $\chi^2 = 0.6$  at 2 df,  $p = 0.7$ ). Control  
16 h (encircled in Figure 2) had a STB which was 2.4 SD  
17 above the mean STB for that group (mean calculated  
18 including control h). The results for control h were  
19 repeatable and he is currently being investigated to  
20 exclude haemochromatosis. Comparison of mean STB in  
21 males and females revealed that females have a  
22 significantly lower concentration than males ( $p = 0.031$   
23 including control h;  $p = 0.0458$  excluding control h).  
24 There was a strong correlation between genotype and  
25 mean STB concentration within the control group ( $p <$   
26  $0.001$ ) irrespective of whether control h was included  
27 and there was a significant difference in mean STB  
28 between males and females of the same genotype ( $p <$   
29  $0.05$ ) irrespective of whether control h was included  
30 (see Figure 2). All patients studied had the 7/77  
31 genotype.  
32  
33 Correlations between STB/PSAT ( $r = 0.4113$ ,  $p =$   
34  $0.001$ ) (see Figure 2) and STB/iron females ( $p = 0.001$ )  
35 than males ( $p = 0.01$ ) but when control h is excluded  
36 there was no significant correlation in males.

1 The STB concentrations of control who underwent the 24  
2 hour restricted diet (see Methods) are shown in Table  
3 1. The normal fasting response is a small rise in the  
4 base-line STB (not exceeding a final concentration of  
5  $25\mu\text{mol/l}$ ) most of which is unconjugated while GS  
6 patients have a lone biochemical feature a raised STB  
7 ( $>25\mu\text{mol/l}$  but  $<50\mu\text{mol/l}$ ) most of which is  
8 unconjugated<sup>14</sup>. The 6/6 and 6/7 controls had post-  
9 fasting STB of  $\leq 23\mu\text{mol/l}$  while all 7/77 controls were  
10  $\geq 31\mu\text{mol/l}$ . Other liver function tests were within  
11 acceptable ranges for the age and sex of the subjects.  
12 The 7/77 genotype correlates with a fasted STB (24  
13 hour) within the range diagnostic for GS<sup>14</sup> ( $p <$   
14  $0.01$ ) (see Table 1). In addition, the 7/7 genotype  
15 segregates with elevated STB concentration in a family  
16 with 4 GS members (Figures 3).  
17  
18 Table 1 shows a comparison of the UGT1\*1 exon 1  
19 genotype with elevation in the serum total bilirubin  
20 after a 24 hour 400-calorie restricted diet<sup>14</sup>.  
21  
22 An elevation of the fasting STB to a final  
23 concentration in the range  $25-50\mu\text{mol/l}$  is considered to  
24 be diagnostic for GS<sup>14</sup>. The 7/7 subject denoted by \*  
25 has a fasting and non-fasting STB of  $> 50\mu\text{mol/l}$  but  
26 this value is within a range considered by others to  
27 conform to a diagnosis of GS<sup>7-11</sup>.

Table 1

Genotype	Sex	24 hour fast		Fasting bilirubin >25 & <50 $\mu$ mol/l
		Before	After	
6/6	M	8	17	NO
	M	9	19	NO
	M	12	15	NO
6/7	F	8	17	NO
	F	9	13	NO
	F	11	12	NO
	F	12	17	NO
	M	8	10	NO
	M	15	23	NO
	M	17	18	NO
7/7	F	9	34	YES
	F	12	34	YES
	M	19	31	YES
	M	62	96	NO*

# 1 Discussion

2

3 A few recent reports claim to have identified the

4 genetic cause of GS<sup>10-12</sup>. Clinical diagnosis of GS is

5 often based on a consistent mildly elevated non-fasting

6 STB (>17  $\mu$ mol/l) as the sole abnormal liver function

7 test, intermittent jaundice or both. The diagnosis can

8 be confirmed by elevation of the STB to 25-50 $\mu$ mol/l

9 after a 24 hour 400-calorie diet<sup>14</sup> or by elevation of

10 the unconjugated bilirubin by > 90% within 48 hours of

11 commencing a 400 calorie diet<sup>13</sup>.

12

13 Sato's research group recently reported the occurrence

14 of 7 different heterozygous missense mutations in

15 unrelated Gilbert patients (most of the mutations have

16 been found in the homozygous state in affected members

17 of CN families), however, the non-fasted STB for these

18 patients were > 52 $\mu$ mol/l (with the exception of one,

1     31 $\mu$ mol/l)<sup>10,12</sup>. These non-fasted STB concentrations  
2     already exceed the diagnostic range for GS<sup>14</sup>, hence  
3     these patients have a more severe form of  
4     hyperbilirubinaemia than those studied in this report,  
5     while those in the Bosma et al<sup>12</sup> abstract had STB  
6     concentrations similar to those studied here.

7  
8     The example herein shows that the variation in the STB  
9     levels after an overnight fast (and in the absence of  
10    exposure to known inducers of the UGT1\*1 isoform in GS,  
11    such as alcoholic<sup>15</sup> and drugs<sup>16</sup>) a representative group  
12    of the Eastern Scottish population is primarily due to  
13    (or associated with) the TATA box sequence variation  
14    reported by Bosma et al<sup>12</sup>. In agreement with previous  
15    work females have a significantly lower mean STB  
16    concentration than males<sup>17,18</sup>.

17  
18    Individuals with the 7/7 genotype in the population  
19    have GS (see Table 1). One of the 7/7 controls  
20    indicated in Table 1 had a non-fasting STB similar to  
21    those reported for heterozygous carriers of CN-2  
22    mutations<sup>7,11</sup> which suggests that this subject may also  
23    be a carrier of a CN-2 mutation, alternatively, the  
24    very elevated bilirubin in this patient may be due to  
25    the coexistence of Reavon's Syndrome (characterized by  
26    a collection of abnormal biochemical results which are  
27    risk factors for coronary heart disease)<sup>19</sup>.

28  
29    We have found that 10-13% of the Eastern Scottish  
30    population have the genotype associated with mild GS.  
31    None of the Gilbert subjects from the control  
32    population were aware that they had an underlying  
33    metabolic defect in glucuronidation with testifies to  
34    its benign nature. Three 7/7 controls had STB  
35    concentrations comparable to mean levels observed in  
36    heterozygotes, however, they also had a lower than

1 normal PSAT ( $\leq 22\%$ ) (see Figure 2). The observed  
2 correlation between STB and PSAT ( $p = 0.001$ ) (Figure 2)  
3 and STB and iron (females  $p = 0.001$  and males  $p = 0.01$   
4 including control h) indicates that other genetic and  
5 environmental factors affecting the serum PSAT and iron  
6 values will in turn affect the STB concentration.

7  
8 From the data presented here and previous reports it  
9 seems clear that there are mild and more severe forms  
10 of GS. The milder form (fasted STB  $25-50\mu\text{mol/l}$ ) is  
11 either caused by (or is associated with) a homozygous  
12 2bp insertion at the TATA sequence upstream of the  
13 UGT1\*1 exon 1 (autosomal recessive inheritance) while  
14 the rarer more severe dominantly inherited forms  
15 identified to date<sup>7-11</sup> (non-fasted STB  $> 50\mu\text{mol/l}$ ) are due  
16 to heterozygosity for a mutation in the coding region  
17 of the UGT1\*1 gene which in its homozygous state causes  
18 CN-2. The particular genetic abnormality causing GS in  
19 a patient will have implications for genetic  
20 counselling as the dominantly inherited form of two GS  
21 patients could result in offspring with CN-2, whereas  
22 the recessive form in one or both GS patients would  
23 have less serious implications. It is important to  
24 discriminate between the two forms and provide suitable  
25 genetic counselling for such couples. The rapid DNA  
26 test presented here (less than 1 day for extracted DNA)  
27 carried out in addition to biochemical tests following  
28 a 12 hour overnight fast (without prior alcohol or drug  
29 intake would permit such a diagnosis. The compliance  
30 rate for the current 24 and 48 hour restricted diet  
31 tests for GS<sup>13-14</sup> is debatable and hence the overnight  
32 fast has obvious advantages and only one blood sample  
33 or a buccal smear is required (for genetic and  
34 biochemical analysis) in contrast to the 2-3 blood  
35 samplings required for the 24 and 48 hour tests. This  
36 approach to GS testing would be cost effective in terms

1 of fewer patient return visits to clinics and in  
2 identifying couples at risk of having children with  
3 CN-2.  
4

5 In addition, the recent finding of an increased  
6 bioactivation of acetaminophen (a commonly used  
7 analgesic which is eliminated primarily by  
8 glucuronidation) in GS patients indicates the greater  
9 potential for drug toxicity in these patients if  
10 administered drugs which are also conjugated by UGT1  
11 isoforms<sup>3</sup>. In fact, ethinylestradiol (EE2) has recently  
12 been shown to be primarily glucuronidated by the UGT1\*1  
13 isoform in man<sup>20</sup> and hence this could have implications  
14 for female Gilbert patients taking the oral  
15 contraceptive who are then more predisposed to  
16 developing jaundice.  
17

18  
19 The tests outlined herein have obvious implications for  
20 setting up drug trials in understanding unusual results  
21 in ruling out individuals who may be adversely affected  
22 by the drugs or in positively choosing these  
23 individuals to determine the effects of particular  
24 drugs on hyperbilirubinaemia.  
25

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## 1 CLAIMS

2

3 1. A method for improving the efficacy of drug  
4 trials, the method comprising the step of  
5 screening samples from potential participants for  
6 the genetic basis of Gilbert's Syndrome and  
7 eliminating or including potential participants in  
8 a drug trial in the knowledge of them possessing  
9 or not possessing the genetic basis of Gilbert's  
10 Syndrome.

11

12 2. A method as claimed in claim 1 comprising the  
13 steps of:

14

15 a) taking a sample from each potential  
16 participant in a drug trial,

17

18 b) screening the samples for the genetic basis  
19 of Gilbert's Syndrome,

20

21 c) identifying participants having the genetic  
22 basis of Gilbert's Syndrome, and

23

24 d) proceeding with drugs trials in the knowledge  
25 of participants possessing or not possessing  
26 the genetic basis of Gilbert's Syndrome.

27

28 3 A method as claimed in claim 1 or 2 wherein the  
29 sample is chosen from blood, buccal smear or any  
30 other sample containing DNA from the potential  
31 participants.

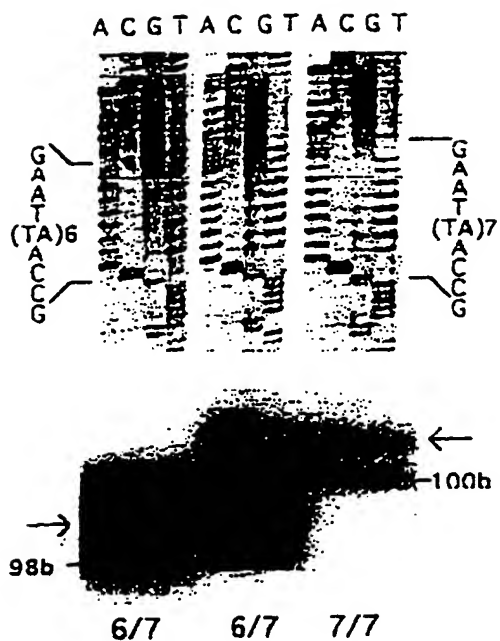
32

33 4. A method as claimed in any of the preceding claims  
34 further comprising the step of eliminating  
35 participants having the genetic basis of Gilbert's  
36 Syndrome from a drugs trial.

- 1     5.    A method as claimed in any of claims 1 to 3  
2            wherein the method comprises the further step of  
3            selecting only participants having genetic basis  
4            for Gilbert's Syndrome for a drugs trial.  
5
- 6     6.    A method as claimed in any of claims 1 to 3  
7            further comprising the step of interpreting the  
8            results of the drugs trial in the knowledge that  
9            certain participants have Gilbert's Syndrome.  
10
- 11    7.    A method as claimed in any of the preceding claims  
12            wherein the method comprises the steps of:  
13
- 14           a)    isolating DNA from each sample,  
15
- 16           b)    amplifying the DNA inner region indicating  
17                 the genetic basis for Gilbert's Syndrome,  
18
- 19           c)    isolating amplified DNA fragments, and  
20
- 21           d)    identifying individuals having the genetic  
22                 basis of Gilbert's Syndrome.  
23
- 24    8.    A method as claimed in any of the preceding claims  
25            wherein the DNA is amplified using the polymerase  
26            chain reaction (PCR) using a radioactively  
27            labelled pair of nucleotide primers.  
28
- 29    10.   A method as claimed in any of claims 7 to 9  
30            wherein the DNA region indicating the genetic  
31            basis of Gilbert's Syndrome is the gene encoding  
32            UDP-glucuronosyltransferase (UGT).  
33
- 34    11.   A method as claimed in any of claims 7 to 10  
35            wherein the DNA to be amplified is in an upstream  
36            promoter region of the UGT 1\*1 exon 1.

- 1      12. A method as claimed in any of claims 7 to 11  
2            wherein the DNA to be amplified includes the  
3            regions between -35 and -55 nucleotides at the 5'  
4            end of UGT 1\*1 exon.  
5
- 6      13. A kit for screening individuals participation in  
7            drug trials, the kit comprising primers for  
8            amplifying DNA in the region of the genome  
9            indicating the genetic basis of Gilbert's  
10          Syndrome.  
11
- 12      14. Primers for use in a method as claimed in any of  
13            the preceding claims including primer pairs, AB or  
14            CD as follows:  
15
- 16                    A/B (A, 5'-AAGTGAAGTCCCTGCTACCTT-3',  
17                    B, 5'-CCACTGGGATCAACAGTATCT-3') or  
18                    C/D (C, 5'-GTCACGTGACACAGTCAAAC-3';  
19                    D 5'-TTTGCTCCTGCCAGAGGTT-3').

1/4



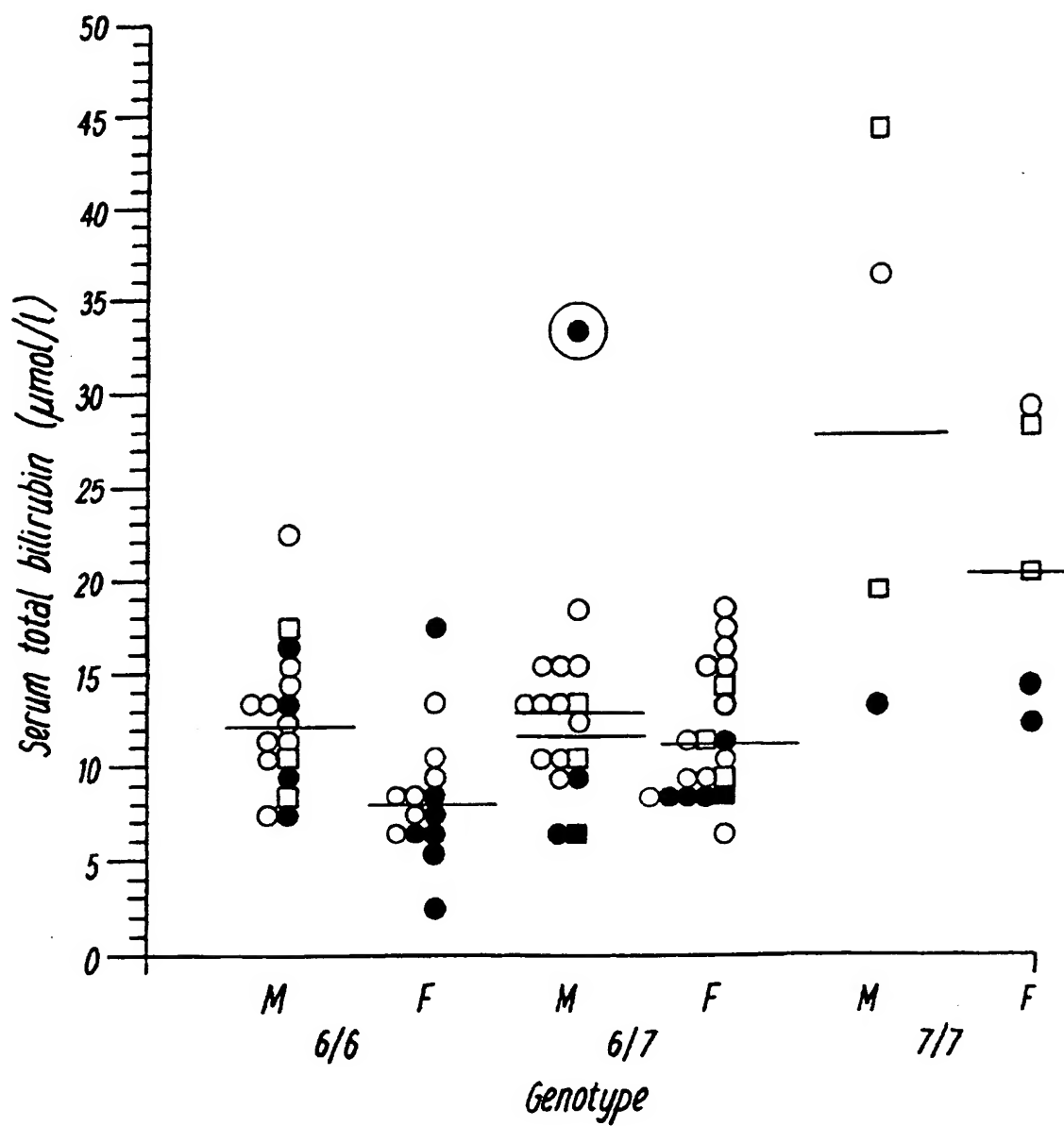
*Fig. 1a*



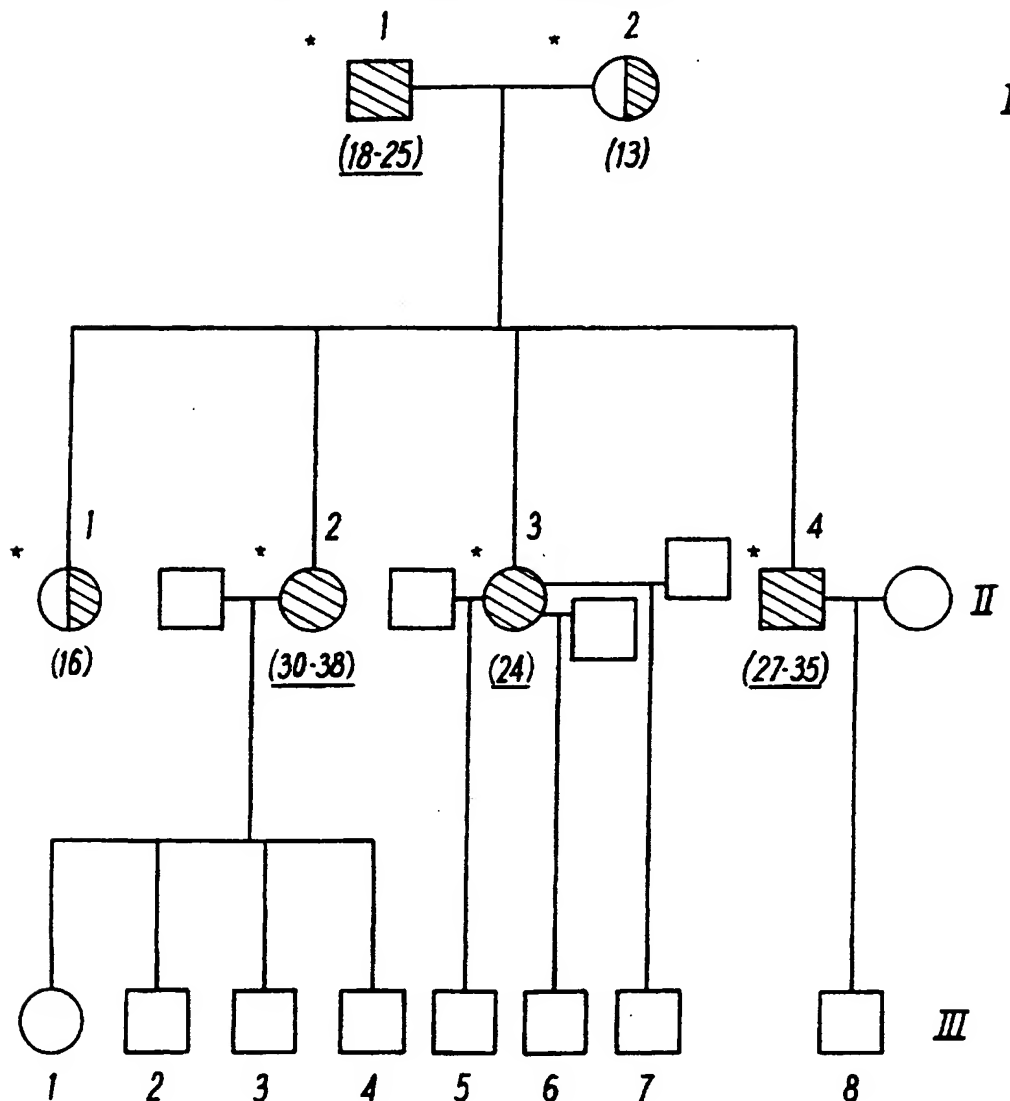
*Fig. 1b*

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**FIG. 2**

*Pedigree Showing Segregation of the Gilbert Phenotype with  $\frac{3}{4}$  the  $(TA)_7TAA / (TA)_7TAA$  Genotype.*



*I, II, III = generations in family    \* = genetic and biochemical data available*

□ male

▨ ▩ homozygotes for the  $(TA)_7TAA$  allele

○ female

⊗ heterozygotes for the  $(TA)_7TAA$  and  $(TA)_6TAA$  alleles

*(13) = total serum bilirubin*

*(18-25) = elevated total serum bilirubin*

**FIG. 3**

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4/4

AP1

-611 GTGAGTCTGGCTCACCTCATGGCGCTGGCTCGTGTGGTGGGCTCTGCTGCAGCCTCCAA

Sp1

-541 GACACCACACTGTGCTGGAAGTCAATAAATAATGTTGGACGAAGGAATGAAACACATGATA

-491 CAAGTGAGCAGGCAGTACCGGGGGAGCTGTGGAGTGGGCACTCTTACAGGTTTCCATGGC

-431 GAAAGCGGGGGGACAGTTGTGTTCTTTTCTTTCTAAAAGGCTTTCTAAAAGCCTTCTGT

-371 TTAATTTCTGGAAAAGAAGCCTAAC TTGTTCACTACATAGTCGTCCTTCTTCCTCTCTGG

AP3

-311 TAACACTTGTGGTCTGTGGAAATACTAATTTAATGGATCCTGAGGTTCTGGAAGTACTT

CLBP

AP1

-251 TGCTGTGTTCACTCAAGAATGTGATTTGAGTATGAAATTCAGCCAGTTCAACTGTTGTT

-191 GCCTATTAAGAAACCTAATAAAGCTCCACCTTCTTTATCTCTGAAAGTGAACCTCCCTGCT

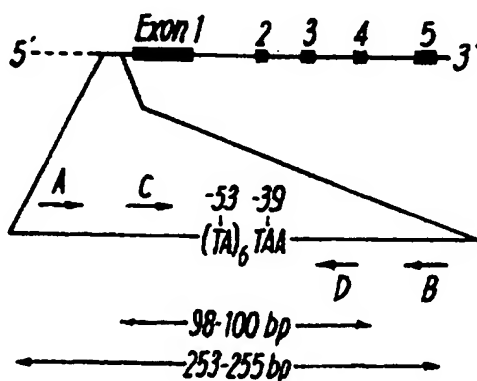
AP1

HNF1

-131 ACCTTTGTGGAAGTGCAGCTTTTATAGTCACGTGACACAGTCAAACATTAAC TTGGTGT

-71 ATCGATTGGTTTTTGGCATATATATATATAAGTAGGAGAGGGCGAACCTCTGGCAGGA

-11 GCAAAGGCCCATGGCTGTG



**FIG. 4**